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Antibody Responses to Natural Rattlesnake Envenomation and a Rattlesnake Toxoid Vaccine in Horses

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Antivenom antibody titers following administration of rattlesnake venom for antivenom production in horses are well documented; however, antivenom antibody titers following natural rattlesnake envenomation in horses are not. Antibody titers produced in response to the commercially available rattlesnake venom vaccine are also not published. Our study objectives were to measure antivenom antibody titers in rattlesnake-bitten horses and compare them to titers in horses vaccinated with the rattlesnake venom vaccine. Additionally, titers were compared in pregnant versus nonpregnant horses to assess the affect of pregnancy on vaccine response and were measured pre- and postsuckle in foals of vaccinated mares to detect passive transfer of vaccine immunoglobulins. Blood samples were collected from16 rattlesnake-bitten horses. Thirty-six horses (11 pregnant mares, 12 nonpregnant mares, 13 geldings) were vaccinated using a *Crotalus atrox* venom toxoid vaccine. Blood was collected before administering each vaccination and 30 days following the third vaccination. Blood was collected from foals of vaccinated mares pre- and postsuckle. All serum was assayed for anti-*Crotalus atrox* venom antibodies using an enzyme-linked immunosorbent assay (ELISA). Rattlesnake-bitten horses had higher ($P = 0.001$) titers than vaccinated horses. There was no significant difference between titers in vaccinated pregnant versus nonpregnant horses. One mare had a positive titer at foaling, and the foals had positive postsuckle titers. Antivenom antibody titer development was variable following natural envenomation and vaccination, and vaccine-induced titers were lower than natural envenomation titers. Further studies are required to determine if natural or vaccine antivenom antibody titers reduce the effects of envenomation.

Antibody titers are frequently measured in horses used for the production of various antivenoms (1). Little is known, however, about antivenom antibody titers produced in horses following natural rattlesnake envenomation or following vaccination with the commercially available rattlesnake venom toxoid vaccine. Information is not available on the duration that antivenom antibody titers persist following natural envenomation and whether or not they protect horses against the adverse effects of venom in subsequent envenomations (2). Clinical signs, laboratory responses, and clinical outcomes following natural rattlesnake envenomation vary in horses, and it is unknown whether differing immune responses play a part in this variability (3–5). In mice, it has been shown that circulating antivenom antibodies present at the time of, or shortly after, experimental envenomation are effective at decreasing the toxic effects of venom (6). In people bitten by the king cobra, there is evidence that the humoral immune response to repeated envenomations is greater, more effective at neutralizing venom effects, and longer lasting than that of a single envenomation (7). Following natural envenomation, the persistence of circulating antibodies is highly variable in people and has been reported to be anywhere from 81 days after a puff adder bite (*Bitis arietans*) (8) to 8 weeks following a king cobra bite (*Ophiophagus hannah*) (7). Antibodies have persisted for 40 years in a patient after being bitten by a black-necked spitting cobra (*Naja nigricollis*) (9). Because people bitten multiple times often have more mild venom effects, vaccination against venom has long been attempted (10, 11). However, snake venoms seem to make poor immunogens, and the duration of immunity is unpredictable (10, 12, 13).

The purpose of this study was to examine the immunologic response to natural rattlesnake envenomation by measuring venom antibody titers in horses bitten by rattlesnakes. Subsequently, we sought to compare immune response to natural envenomation with that stimulated by a commercial rattlesnake toxoid vaccine. The immunologic response to vaccination in late-gestation mares and colostral transfer of antivenom antibodies in foals were also investigated.

(Portions of these data were presented at the 2011 ACVIM Forum in Denver, CO.)

MATERIALS AND METHODS

Study population and data collection. This was a prospective observational study, including two distinct populations of horses. Sixteen horses that presented to participating veterinary clinics with a clinical diagnosis of rattlesnake bite were enrolled in the study. Areas where these horses resided when bitten were the Texas Panhandle (13), western Oklahoma (1), southeast Oklahoma (1), and central Oklahoma (1). The most common snakes in the area of the Texas panhandle and western Oklahoma

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TABLE 1 Demographics of study populations[a]

No. of horses	Mean age (yrs)	Sex	Study group
6	2.5	Fe	NE
6	6.8	G	NE
1	NR	Fe	NE
1	NR	G	NE
2	NR	NR	NE
13	13.6	G	V
12	11.8	Fe	V
11	13.8	Fe/P	V

[a] Fe, female; G, gelding; Fe/P, pregnant female; NE, naturally envenomated horses; V, vaccinated horses; NR, not reported.

where 14 horses were located when bitten are the western diamondback rattlesnake (*Crotalus atrox*) and the prairie rattlesnake (*Crotalus viridis viridis*). The rattlesnakes most likely to reside in the area where the horse was bitten in southeast Oklahoma are the pigmy rattlesnake (*Sistrurus miliarius streckeri*) and timber rattlesnake (*Crotalus horridus horridus*). The most common rattlesnakes residing in the area of central Oklahoma where one horse resided are the pygmy rattlesnake and the timber rattlesnake, with the western diamondback rattlesnake being a less likely candidate. There were 7 nongravid mares and 7 geldings. The mean age was 4.67 years (median, 4 years). (Table 1). The gender and age were not reported for two horses, and two horses had gender but not age reported. Serum samples were collected at the time of presentation, 11 days and 1 month following presentation, and frozen at −80°C until analysis was performed. All horses had samples collected at presentation; however, not all horses were available for both the 11-day and 1-month samples (Table 2).

Thirty-six healthy adult horses that are part of the university research herd were enrolled in the toxoid vaccination portion of the study. These horses had been owned by the university for a minimum of 1 year and had not been bitten by a rattlesnake during this time. The exposure to rattlesnakes prior to that time is unknown. These included 11 late-gestation mares (>270 days), 12 nonpregnant mares, and 13 geldings. Ages ranged from 4 to 20 years, with a mean age of 13.1 years (median, 13.5 years) (Table 1). Each horse received three doses (2 ml) of a commercially available rattlesnake toxoid vaccine (Red Rock Biologics, Woodland, CA) in the pectoral muscle at 30-day intervals. Serum samples were obtained prior to administration of each vaccination and 30 days following the last vaccination. All horses were monitored for adverse vaccine reactions. Overall demeanor was observed, and the vaccination site was palpated 24 h after each vaccination. A visual examination was performed on days 7, 14, and 21 after each vaccination. All late-gestation mares were followed to parturition, and the status of the foal at birth was noted. Serum was collected from foals prior to their first suckle and at 24 h of age. Samples were not available for 3 foals. All serum samples were stored at −80°C until assayed. Horses with positive antivenom antibody titers prior to the first vaccine were excluded from the study.

This study was approved by the Institutional Animal Care and Use Committee at Oklahoma State University, and all legal and ethical guidelines for humane care were followed.

Venom antibody ELISA. Serum samples were assayed for antibodies against crude *Crotalus atrox* venom using standard direct enzyme-linked immunosorbent assay (ELISA) techniques (14). Anti-equine alkaline phosphatase antibody (Sigma, St. Louis, MO) was used as a detection antibody. Plates were read at 405 nm with a spectrophotometer (THERMOmax microplate reader; Molecular Devices, Sunnyvale, CA), and titers were calculated using a standard curve. A positive titer was the minimum titer that would neutralize *Crotalus atrox* venom based on a cell culture and mouse inoculation model. These venom neutralization experiments were performed during product development of the rattlesnake toxoid vaccine (unpublished data). Titers greater than 1:200 were considered positive.

TABLE 2 Sample timing and antivenom antibody titers of rattlesnake-bitten horses

Horse	Time postpresentation of sample collection	Antivenom antibody titer
1	0	130
	11 days	1,370
	1 mo	515
2	0	105
	11 days	140
	1 mo	245
3	0	100
	11 days	825
4	0	95
	1 mo	2,825
5	0	100
	1 mo	5,860
6	0	85
	1 mo	9,975
7	0	110
	11 days	120
8	0	120
	11 days	125
	1 mo	1,435
9[a]	0	130
10	0	105
	11 days	5,570
11	0	120
	11 days	295
12[a]	0	4,250
	11 days	17,060
13	0	135
	11 days	925
	1 mo	1,620
14	0	120
	11 days	170
15	0	115
	1 mo	6,130
16	0	95
	11 days	33,600

[a] Horses 9 and 12 presented 1 week after being bitten.

Data analysis. In order to correct for the lack of normality and homogeneous variances, the data were transformed with a square root function. A Student *t* test was used to compare the venom antibody titers of horses naturally envenomated to those receiving the rattlesnake toxoid vaccine. Fisher's exact test was used to assess differences in categorical titer levels of vaccinated late-gestation pregnant mares versus nonpregnant vaccinated horses (mares and geldings). Fisher's exact test was also used to detect differences between the number of vaccinated pregnant mares that had a 2-fold or greater increase in their venom antibody titer and the number of vaccinated nonpregnant horses that had a similar increase.

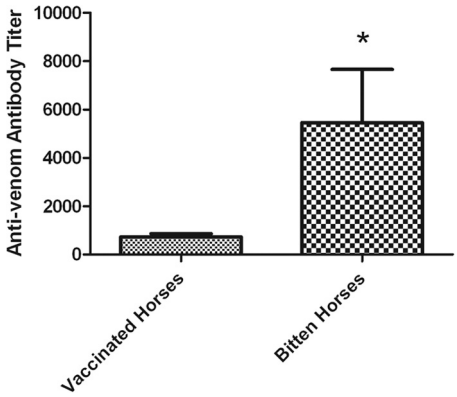

FIG 1 Peak antivenom antibody titers in vaccinated versus bitten horses.

TABLE 3 Presuckle and 24 h after suckle antivenom antibody titers in foals born to vaccinated mares[a]

Horse	No. of vaccines given to mare prior to parturition	Mare's titer at parturition	Foal's presuckle titer	Foal's 24-h postsuckle titer
28	3	120	95	125
30	2	375	105	280
31	3	200	110	185
32	2	125	75	175
35	1	100	80	95
36	1	125	90	140
37	1	195	90	115
39	2	125	105	105

[a] Gray boxes indicate mare with positive titer at parturition.

RESULTS

Venom antibody titers were measured at presentation in 16 horses with a clinical diagnosis of rattlesnake bite. Follow-up titers were measured at 11 days in 7 horses, 1 month in 4 horses, and both 11 days and 1 month in 4 horses. One horse was not available for a follow-up sample. Time after bite to initial presentation was <24 h in 14 horses and 1 week in 2 horses. All horses that presented within 24 h of the bite had negative titers upon presentation. Five horses had negative titers at 11 days postenvenomation. Three of these five horses did not have 1-month samples available for assay. The two that had 1-month samples assayed had positive titers at 1 month postpresentation. In horses where titers were measured at both 11 days and 1 month, the highest titer was used for statistical comparisons (Table 2).

Venom antibody titers were measured in 36 healthy research horses at four time points, prior to the first vaccination and 30 days after receiving each of three doses of the rattlesnake toxoid vaccine. Peak titers were used for statistical comparisons. Horses bitten by a rattlesnake had significantly higher peak antibody titers than horses receiving the rattlesnake toxoid vaccine ($P = 0.001$) (Fig. 1). Ten of 36 horses (28%) showed no response to the vaccine series; 3 of them were pregnant mares. Two horses developed a peak titer 30 days after the first vaccination, 9 horses had a peak titer 30 days after the second vaccination, and 15 horses developed peak titers 30 days after the third vaccination (Fig. 2). Thirty days after the last vaccination, 15 horses had increasing titers, while 11 horses' titers were decreasing or unchanged. Eleven of the vaccinated horses were mares in late gestation, and all of them gave birth to live, healthy foals. There was no difference in peak titers between nonpregnant horses (mares and geldings) and pregnant mares at any time point. Pre- and postsuckle serum samples were available from 8 foals born to vaccinated mares. The mares were at various stages in the vaccine series at foaling. Only one of the mares had a positive titer prior to foaling. The foal born to this mare had a positive postsuckle titer (Table 3).

All vaccinated horses were monitored for adverse vaccine reactions. Generalized enlargement of the pectoral muscle was noted in three horses, and a firm knot at the injection site was noted in two horses. The injection site reactions in these five horses were monitored daily, and they all resolved within 21 days without treatment. No changes in attitude or appetite were noted in any of the vaccinated horses.

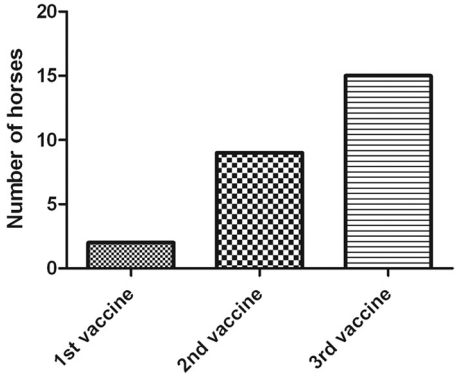

FIG 2 Number of vaccinated horses with peak antivenom antibody titers 30 days after each vaccine dose.

DISCUSSION

Horses have long been used for the production of commercial antivenoms. The production of antibodies in horses vaccinated with crude rattlesnake venom and venom components is well studied (1, 2, 15–17); however, the antibody response of horses to natural rattlesnake envenomation is not well documented. One goal of this study was simply to document antibody titers in naturally envenomated horses and to document the presence or absence of preexisting venom antibody titers. None of the horses in this study had antibody titers within 24 h of being bitten. Documentation of previous envenomation was not available for these horses. The absence of positive titers less than 24 h postbite in these horses was most likely because this was their first exposure to rattlesnake venom. Alternatively, the time since previous exposure to rattlesnake venom could have exceeded the half-life of the venom antibodies. There are various reports of the duration of venom antibody titers in humans (7–9, 18, 19) following natural snake envenomation. In goats experimentally envenomated with *Crotalus atrox* venom, antibodies were short-lived (~60 days) (13). Horses used for snake antivenom production must be repeatedly immunized in order to maintain adequate titers (20). Repeated immunization protocols range from every 2 days (16) to every 2 weeks (20), and there is a large variation in response among individual horses (1). Therefore, it is possible that venom antibody titers following natural envenomation do not persist for prolonged periods of time. Three horses had negative postenvenomation titers; however, they did not have a sample drawn 1 month

postenvenomation. There are a couple possible reasons for these negative titers in the face of clinical signs of rattlesnake envenomation. It is possible, and most likely, that this was these horses' first exposure to rattlesnake venom and 11 days was too early to detect a rise in titer. The immune response to venom is dependent upon the structure and molecular mass of the venom toxins and their relative abundance in venom, the dose of venom administered, and the host's ability to recognize the venom as foreign (21). The hosts ability to recognize the venom as foreign may depend on the route by which it enters the body, the animal's ability to process the venom toxins, and the genetic background of the individual horses (21). In humans bitten for the first time by *Bothrops jararaca*, the increase in IgG occurred at 18 days postenvenomation (18), while those bitten two or more times developed titers by day three (18). It is also possible that these horses received a dry bite or a very small dose of venom that was enough to cause local inflammation but not enough to result in a humoral immune response. During the production of antivenom, it is frequently noted that there can be marked differences in an individual animal's response to venom (21, 22). It is possible that although these horses were envenomated, they are not capable of mounting a humoral immune response against the venom. Additionally, there is a possibility that the assay used in this study was not able to detect venom antibodies to the species of rattlesnake that had bitten these horses. These horses came from areas of Oklahoma and Texas where several rattlesnake species reside, along with the broad-banded copperhead (*Agkistrodon contortrix laticinctus*) and the water moccasin (*Agkistrodon piscivorus*). None of the snakes that bit these horses were positively identified. Although the assay used in this study was designed to detect antibodies against Western diamondback rattlesnake (*Crotalus atrox*) venom, cross-reactivity to multiple rattlesnake venoms has been demonstrated (23, 24). Strength of cross-reactivity varies depending on the species being compared; thus, if a horse was bitten by a snake with weak cross-reactivity with *Crotalus atrox*, this assay may not detect a positive antivenom antibody titer (23). While this is possible, it is unlikely to be the cause of the negative titers (25). Finally, the effect of storage on antibody decomposition must be considered. Long-term storage of serum samples at −20°C has been shown to decrease measured immunoglobulin G concentrations (26). However, storage at −80°C, as was done in this study, is considered the standard for samples to be assayed for immunoglobulin concentration and has not been associated with a decrease in immunoglobulin concentrations in other species (27, 28); therefore, it is unlikely that this affected antibody concentrations in our study.

A second aim of this study was to compare titers of naturally envenomated horses to those of horses vaccinated with a commercially available rattlesnake toxoid vaccine. Titers in the naturally envenomated horses were significantly higher than those in the vaccinated horses. This could be due to a higher antigen load in the naturally envenomated horses or due to a reduced immune response to the detoxified venom in the vaccine. One primary disadvantage of immunization with a detoxified venom is the risk of losing epitopes that are important in the immunogenicity of the venom (21). Approximately 28% of the vaccinated horses did not respond to the rattlesnake vaccine. This is not surprising based on the individual animal variability that is seen in horses that are given crude venom during the production of antivenoms (20, 21). The anamnestic response to this commercial vaccine was also quite variable considering approximately half of the horses had increasing titers 30 days after the third vaccine booster while half had decreasing titers. The manufacturer's recommendation is to give boosters every 6 months after the initial series of three doses given 30 days apart. It may be prudent to consider revising this dosing schedule to booster the animal more frequently during the rattlesnake season and not administer the vaccine during times when the horse is highly unlikely to encounter a rattlesnake in order to maintain peak titers during peak exposure times.

Eleven of the horses that were vaccinated were pregnant mares. Pregnancy has been shown to downregulate the Th1 response of the immune system and shift it more toward a Th2 response (29). This would typically promote a more robust humoral immune response; however, we did not detect a difference in the overall antibody titers in the pregnant mares versus the nonpregnant horses (mares and geldings).

Foals are immunocompetent at birth, but their immune systems have not been challenged (30). Since the mare's placentation is epitheliochorial, foals do not receive immunoglobulins *in utero* and are completely dependent on colostral transfer for immunoglobulins for the first weeks of life (30). Mare's colostrum contains immunoglobulin G as well as immunoglobulin A, and foals receive these immunoglobulins through enteric absorption within the first 24 h of life (31, 32). Pre- and postsuckle serum samples were available only for foals from 8 of the 11 vaccinated mares, and only 1 of these mares had a positive titer at parturition. The foal from the mare with a positive titer had a negative presuckle titer and a positive titer at 24 h of age. This result suggests that colostral transfer of venom antibodies produced in response to the toxoid vaccine may be possible. All the foals were born healthy, and no adverse vaccine reactions were noted in the mares, indicating the vaccine is safe to be given to late-gestation mares.

We hypothesized that the variability in clinical response to rattlesnake envenomation in horses may be due to preexisting antibody titers in individuals that had been previously bitten. The absence of preexisting antibodies in this population of horses indicates the clinical variability was not due to preexisting antibody titers; however, this may not be true in all populations. In humans experiencing snake bite, there is evidence that preexisting titers may protect against death but not against the local venom effects (33–35). A person bitten by a death adder (*Acanthophis* sp.) that had a preexisting antibody titer from a previous bite did not experience the typical neurotoxic signs, although they experienced the local tissue effects of the venom (34). Horses bitten by rattlesnakes typically experience significant local tissue effects but can also have systemic effects, including cardiac damage (3–5). Similar to this human case, preexisting antibodies whether from vaccination or natural envenomation may not prevent local tissue effects but may have protective effects on other systemic effects, such as cardiotoxicity. In most cases of envenomation, the onset of the toxic effects of the venom are extremely rapid, and antibodies would have to be in circulation at the time of envenomation to make a difference (33); however, in some horses that are bitten by rattlesnakes, evidence of cardiotoxicity is not seen for several days after envenomation (3). The reason for this delay is unknown; however, it has been hypothesized that it could be due in part to a delay in venom release from the tissues at the bite site. Preexisting antibodies and an anamnestic response to venom exposure may be particularly helpful in these cases. Venom neutralization is not an all-or-none phenomenon. Any amount of venom that is neu-

tralized is effectively removed from the circulation, and therefore there is less to exert toxic effects on the animal. Interestingly, three horses were excluded from this study due to positive antivenom antibody titers prior to receiving the rattlesnake vaccine. These horses all received the vaccine series, and each of these horses had what would be considered a robust response to the vaccine, with titers greater than 1,000. These horses were donated to our university, and no history could be gathered on them as far as previous rattlesnake exposure. It is likely that they had been previously bitten; however, we could not rule out the possibility of a nonspecific cross-reactivity whereby antibodies not specific to rattlesnake venom reacted with the venom in this ELISA.

Antibodies that develop as a result of natural envenomation may be functionally distinct from those produced in response to a rattlesnake toxoid vaccine. As previously mentioned, one major disadvantage to immunization with detoxified venom is the risk of losing important epitopes and therefore having decreased immunogenicity. Others have demonstrated that this can result in the inability of antibodies made against detoxified venom to neutralize native venom (21). However, *in vitro* studies performed during the licensing of the product we used for vaccination demonstrated venom neutralization with serum from horses vaccinated with the rattlesnake toxoid vaccine in a cell culture model as well as a murine model (unpublished data). In the cell culture model, horses were vaccinated and then serum was harvested. Serial dilutions of serum and *Crotalus atrox* venom were added to cell culture, replacing the cell culture media. Cellular viability was then measured. In the murine model, serum from vaccinated horses and control horses was mixed with *Crotalus atrox* venom. Mice were injected intraperitoneally with either a venom-vaccinated horse serum mixture or a venom-control horse serum mixture, and lethality was measured over a 72-h period. Cell survival was increased more than 2-fold in the cells incubated with the serum-venom mixture from vaccinated horses compared to the control. All mice given the serum-venom mixture from vaccinated horses survived, while all mice given the serum-venom mixture from control horses died. These studies indicate venom neutralization by the antibodies in vaccinated horses; however, challenge studies in vaccinated horses have not been performed.

In conclusion, while horses develop anti-*Crotalus atrox* antibody titers to the commercially available rattlesnake toxoid vaccine, they are not as high as those that develop after natural rattlesnake envenomation. The humoral immune responses of individual horses to the vaccine and natural envenomation varied greatly. The commercially available rattlesnake toxoid vaccine was safe when administered to late-gestation mares, and there is evidence that colostral transfer of venom antibodies will occur. Based on existing literature, circulating titers may offer some protection against the systemic effects of envenomation; however, the ability of these titers to protect horses from the adverse effects of envenomation has not been tested *in vivo*.

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REFERENCES

1. **Sriprapat S, Aeksowan S, Sapsutthipas S, Chotwiwatthanakun C, Suttijitpaisal P, Pratanaphon R, Khow O, Sitprija V, Ratanabanangkoon K.** 2003. The impact of a low dose, low volume, multi-site immunization on the production of therapeutic antivenoms in Thailand. Toxicon **41**:57–64.
2. **Angulo Y, Estrada R, Gutierrez JM.** 1997. Clinical and laboratory alterations in horses during immunization with snake venoms for the production of polyvalent (Crotalinae) antivenom. Toxicon **35**:81–90.
3. **Gilliam LL, Holbrook TC, Martin S, Giulioli-Royce AA, Payton ME, Ownby CL.** 2012. Cardiotoxicity, inflammation, and immune response after rattlesnake envenomation in the horse. J. Vet. Intern. Med. **26**:1457–1463.
4. **Dickinson CE, Traub-Dargatz JL, Dargatz DA, Bennett DG, Knight AP.** 1996. Rattlesnake venom poisoning in horses: 32 cases (1973–1993). J. Am. Vet. Med. Assoc. **208**:1866–1871.
5. **Fielding CL, Pusterla N, Magdesian KG, Higgins JC, Meier CA.** 2011. Rattlesnake envenomation in horses: 58 cases (1992–2009). J. Am. Vet. Med. Assoc. **238**:631–635.
6. **Rucavado A, Lomonte B.** 1996. Neutralization of myonecrosis, hemorrhage, and edema induced by Bothrops asper snake venom by homologous and heterologous preexisting antibodies in mice. Toxicon **34**:567–577.
7. **Tun-Pe, Aye-Aye-Myint, Warrell DA, Tin-Myint.** 1995. Warrell, Tin-Myint, king cobra (Ophiophagus hannah) bites in Myanmar: venom antigen levels and development of venom antibodies. Toxicon **33**:379–382.
8. **Theakston RDG.** 1985. Venom antibody levels in a patient bitten by a young puff adder (Bitis arietans) during a world record attempt. Ann. Trop. Med. Parasitol. **79**:305–307.
9. **Theakston RDG, Pugh RNH, Reid HA.** 1981. Enzyme-linked immunosorbent assay of venom antibodies in human victims of snake bite. J. Trop. Med. Hyg. **84**:109–112.
10. **Wiener S.** 1960. Active immunization of man against the venom of the Australian tiger snake. Am. J. Trop. Med. Hyg. **9**:284–292.
11. **Russell FE.** 1988. Snake venom immunology: historical and practical considerations. J. Toxicol. Toxin Rev. **7**:1–82.
12. **Oliveira NJF, Melo MM, Lara ER, Lucia M, Lobato ZIP.** 2007. Clinical and immunological characteristics of cattle experimentally inoculated with crude venom and iodinated Bothrops alternatus. Braz. Arch. Vet. Med. **59**:569–576.
13. **Glenn WG, Becker RE, De Buysere M.** 1970. Goat antibody response to rattlesnake venom during and after immunization. Am. J. Vet. Res. **31**:1237–1240.
14. **Crowther JR.** 2009. Systems in ELISA, p 16–19. *In* Walker JM (ed), The ELISA guidebook, 2nd ed. Humana Press, New York, NY.
15. **Lavonas EJ.** 2012. Antivenoms for snakebite: design, function and controversies. Curr. Pharmaceut. Biotechnol. **13**:1980–1986.
16. **Freitas TV, Fortes-Dias CL, Diniz CR, Velarde DT, Freitas CF.** 1991. Immunization of horses with Crotalus durissus terrificus (South American rattlesnake) venom. A comparison of four different procedures. Braz. J. Med. Biol. Res. **24**:281–290.
17. **Theakston RDG.** 1989. New techniques in antivenom production and active immunization against snake venoms. Trans. R. Soc. Trop. Med. Hyg. **83**:433–435.
18. **Domingos MO, Cardoso JL, Moura da Silva AM, Mota I.** 1990. The humoral immune responses of patients bitten by the snake Bothrops jararaca (jararaca). Toxicon **28**:723–726.
19. **Theakston RDG, Reid HA, Larrick JW, Kaplan J, Yost JA.** 1981. Snake venom antibodies in Ecuadorian Indians. J. Trop. Med. Hyg. **84**:199–202.
20. **Guidolin RG, Marcelino RM, Gondo HH, Morais JF, Ferreira RA, Silva CL, Kipnis TL, Silva JA, Fafetine J, da Silva WD.** 2010. Polyvalent horse $F(Ab')_2$ snake antivenom: development of a process to produce polyvalent horse $F(Ab')_2$ antibodies anti-African snake venom. Afr. J. Biotechnol. **9**:2446–2455.
21. **Leon G, Sanchez L, Hernandez A, Villalta M, Herrera M, Segura A, Estrada R, Gutierrez JM.** 2011. Immune response towards snake venoms. Inflamm. Allergy Drug Targets **10**:381–398.

22. **Villalta M, Pla D, Yang SL, Sanz L, Segura A, Vargas M, Chen PY, Herrera M, Estrada R, Cheng YF, Lee CD, Cerdas M, Chiang JR, Angulo Y, Leon G, Calvete JJ, Gutierrez JM.** 2012. Snake venomics and antivenomics of Protobothrops mucrosquamatus and Viridovipera stejnegeri from Taiwan: keys to understand the variable immune response in horses. J. Proteomics **75**:5628–5645.
23. **Kornalik F, Taborska E.** 1989. Cross reactivity of mono- and polyvalent antivenoms with Viperidae and Crotalidae snake venoms. Toxicon **27**: 1135–1142.
24. **Berger BJ, Bhatti AR.** 1989. Snake-venom components and their cross-reactivity—a review. Biochem. Cell Biol. **67**:597–601.
25. **Ownby CL, Colberg TR.** 1990. Comparison of the immunogenicity and antigenic composition of several venoms of snakes in the family crotalidae. Toxicon **28**:189–199.
26. **Davis DG, Schaefer DMW, Hinchcliff KW, Wellman ML, Willet VE, Fletcher JM.** 2005. Measurement of serum IgG in foals by radial immunodiffusion and automated turbidometric immunoassay. J. Vet. Int. Med. **19**:93–96.
27. **Pinsky NA, Huddleston JM, Jacobson RM, Wollan PC, Poland GA.** 2003. Effect of multiple freeze-thaw cycles on detection of measles, mumps, and rubella virus antibodies. Clin. Diagn. Lab. Immunol. **10**: 19–21.
28. **Neumann EJ, Bonistalli KN.** 2009. Effect of blood sample handling post-collection on Erysipelothrix rhusiopathiae antibody titres. Vet. J. **180**:325–329.
29. **Noronha LE, Antczak DF.** 2010. Maternal immune responses to trophoblast: the contribution of the horse to pregnancy immunology. Am. J. Reprod. Immunol. **64**:231–244.
30. **Fenger CK.** 1998. Neonatal and perinatal diseases, p 941. *In* Reed SM, Bayly WM (ed), Equine internal medicine. W.B. Saunders, Philadelphia, PA.
31. **Horohov DW.** 1998. The equine immune system, p 7. *In* Reed SM, Bayly WM (ed), Equine internal medicine. W.B. Saunders, Philadelphia, PA.
32. **Sheoran AS, Timoney JF, Holmes MA, Karzenski SS, Crisman MV.** 2000. Immunoglobulin isotypes in sera and nasal mucosal secretions and their neonatal transfer and distribution in horses. Am. J. Vet. Res. **61**: 1099–1105.
33. **Wiener S.** 1961. Snake bite in a subject actively immunized against snake venom. Med. J. Australia **48**:658–659.
34. **Isbister GK, Halkidis L, O'Leary MA, Whitaker R, Cullen P, Mulcahy R, Bonnin R, Brown SGA.** 2010. Human anti-snake venom IgG antibodies in a previously bitten snake-handler, but no protection against local envenoming. Toxicon **55**:646–649.
35. **Theakston RDG, Reid HA, Iddon D.** 1983. Protective effect of snake venom antibodies in sera of previous snake bite victims. Ann. Trop. Med. Parasitol. **77**:311–314.